Plant Cell Tiss Organ Cult (2017) 129:119–132
DOI 10.1007/s11240-016-1161-8

ORIGINAL ARTICLE

Identification of *LEC1, L1L* and *Polycomb Repressive Complex 2* genes and their expression during the induction phase of *Medicago truncatula* Gaertn. somatic embryogenesis

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Received: 14 October 2016 / Accepted: 18 December 2016 / Published online: 29 December 2016

Abstract Although somatic embryogenesis (SE), a multi-step process starting from somatic tissues and ending with somatic embryos, has been applied to numerous plants including *Medicago* sp., the molecular basis of development reprograming in somatic cells toward the embryogenic pathway is still incompletely known. Though recent analysis of the proteome and transcriptome has led to the identification and characterization of new genes involved in SE, lot of these genes are up-regulated only in the late developmental stages. Consequently, this work was aimed at finding out if and when the genetic program changed during the SE induction phase in both the highly embryogenic line M9-10a of *Medicago truncatula* cv. Jemalong and its non-embryogenic predecessor line, M9. Based on multi-point (day 0, 2, 7, 14 and 21) gene expression qPCR analysis of two embryogenesis marker genes *LEC1* and *L1L* and selected genes encoding proteins of PRC2 complex (*CLF, SWN, FIE, MSI1, VRN2)* it was possible to distinguish two periods during the induction phase. The first week was related to dedifferentiation with no visible changes in explant morphology and lack of transcripts of *LEC1* and *L1L*; the expression of PRC2 members, however, increased in the embryogenic line. The next two weeks were regarded as the expression phase involving the beginning of a rapid callus growth and the appearance of products for *LEC* genes, which was observed in the embryogenic line only. However, the callus formation was observed in the non-embryogenic line as well, but the *LEC1* and *L1L* genes were not expressed and the transcription of *PRC2* genes was at stable level. It was only the *SWN* expression that decreased at the beginning of induction and did not change in the subsequent days in both lines. Our results indicate that *LEC1* and *L1L*, known as marker genes of the late developmental stages, may be associated with the acquisition of embryogenic competency by somatic cells (prime events in the SE induction). The *PRC2* complex genes are also expressed during embryogenic callus tissue formation on *Medicago tuncatula* leaf explants.

Keywords Expression pattern · Induction of somatic embryogenesis · Legumes · Epigenetic regulation

Abbreviations

CLF	Curly Leaf
FIE	Fertilization-Independent Endosperm
L1L	Leafy Cotyledon1-like
LEC1	Leafy Cotyledon1
MSI1	Multicopy Suppressor of Ira1
PRC2	Polycomb Repressive Complex2
SE	Somatic embryogenesis
SWN	Swinger
VRN2	Vernalization2

Introduction

Somatic embryogenesis (SE) is a multi-step in vitro regeneration process in which embryos are formed from somatic cells without the fusion of gametes (Williams and Maheswaran 1986; Zimmerman 1993). To date, such vegetative propagation techniques have been regarded as

Electronic supplementary material The online version of this article (doi:10.1007/s11240-016-1161-8) contains supplementary material, which is available to authorized users.

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the lowest-cost methods for producing uniform, identical plants, particularly of those species that are difficult to propagate in a natural way or in other conventional manner. The process is also used to study the embryonic development regulation at morphological, biochemical, genetic and epigenetic levels (Willemsen and Scheres 2004; Elhiti et al. 2013; Smertenko and Bozhkov 2014). Competence to somatic embryogenesis is highly correlated with the genotype. At last two embryogenic lines, 2HA and M9-10a, are known in *Medicago truncatula* (Nolan at al. 1989; Neves et al. 1999) and are considered as models for study SE in this species. M9-10a line was directly derived from non-embryogenic (M9) as effect of somaclonal variation. This is a good model to compare progress of SE without potential background that result from comparison different genetic origin lines (Almeida et al. 2012). Generally, SE takes a few weeks starting from a single cell or a cell group of somatic explants. Its fundamental phases are distinguished at the morphological level, and are divided into a number of stages: induction of proembryogenic structures, followed by embryo formation, their maturation and dehydratation. SE terminates with embryos ready to regenerate a new plant. Each of these phases is regulated by various intrinsic (e.g. developmental stage of the starting explant and hormone levels) as well as extrinsic factors. The latter one particularly a number of physical and chemical treatments applied at appropriate schedules seems to be the critical for successful and efficient somatic embryogenesis. However, among all these factors, plant growth regulators appear to play crucial role in SE. The 2,4-D artificial auxin, the popular SE inductor, is sufficient in many species to initiate a genetic program that leads to the establishment of cell lineages with an altered gene transcription pattern, and a different morphology and developmental fate (Gaspar et al. 1996; Karami et al. 2009). Different groups of genes are known to be expressed and essential during somatic embryogenesis, including *WUSCHEL* (*WUS*; Zuo et al. 2002), *AGAMOUS LIKE-15* (*AGL15*; Harding et al. 2003; Zheng et al. 2013), *BABY-BOOM* (*BBM;* Boutilier et al. 2002) and *LEAFY COTYLEDON* (*LECs*, i.e. *L1L, LEC1, LEC2, FUSCA3;* Gaj et al. 2005; Ledwoń and Gaj 2011).

The *LEC* genes are generally known to play multiple roles during zygotic embryogenesis in the early and late phase of embryonic development when the molecular and cellular environment necessary for the process is being established (Jia et al. 2013). Expression of *LEC1* and its close homolog *L1L* plays an important role in zygotic embryogenesis regulating embryo identity and development (Kwong et al. 2003). The role of these genes is mainly to control seed maturation, to inhibit premature germination, and to identify embryonic organs (Meinke 1992; West et al. 1994). *LEC*s are also considered as belonging to the somatic embryogenesis marker genes. One of them, *LEC1*, is a CCAAT-binding (CBF) transcription factor with a HAP3 subunit (Lee et al. 2003). The *LEC1* genes expression pattern was analyzed during somatic embryogenesis from different initial explants representing different genetic program such as immature embryos of *Zea mays* and *Arabidopsis thaliana* (Zhang at al. 2002; Gaj et al. 2005), *Coffea canephora* seedling leaves (Nic-Can et al. 2013), *Daucus carota* hypocotyl (Yazawa et al. 2004), and *Medicago sativa* protoplasts (Domoki et al. 2006). LEAFY COTYLEDON 1-LIKE B-domain of HAP3 shared 83% similarity to that of LEC1, which means that they define distinct class of HAP3 subunits (Kwong et al. 2003). The *L1L* is crucial for proper embryogenesis performance. Its expression was analyzed during SE induction from single-node *Helianthus annuus* (Fambrini et al. 2006), *Theobroma cacao* staminodes (Alemanno et al. 2008) as well as the stamen and nodal *Vitis vinifera* (Schellenbaum et al. 2008; Maillot at al. 2009). Overexpression of the *LEC1* gene in *Arabidopsis* induced the embryonic process and expressed a set of genes important for embryonic development (Lotan et al. 1998). On the other hand, *L1L* is not regarded as a SE marker, but its overexpression could rescue *lec1* mutant and it is up-regulated by *LEC2*, another key SE transcription factor (Guo et al. 2013).

Epigenetic regulation can be another important player during SE induction, capable of controlling genome-wide changes in gene expression during the cell fate transition from a somatic explant (e.g. leaf blade) to the embryogenic callus (He et al. 2012). To date, three levels of epigenetic control of SE are known: the micro RNA (miRNA) pathway, the DNA methylation, and histone post-translational modifications (Smertenko and Bozhkov 2014). Histone modifications are driven by the Polycomb group proteins (PcG) and are essential for cell fate determination, cellular differentiation and transition through successive stages of development from seed to seed in plants (Hennig and Derkacheva 2009; Bemer and Grossniklaus 2012). In plants they form two main conserved protein complexes: the Polycomb Repressive Complex 1 (PRC1) and the well-characterized PRC2. PRC2 catalyzes trimethylation of histone 3 (H3K27me3) lysine 27 through the SET-domain protein. PRC1 binds to the H3K27me3 and ubiquitinates lysine 119 of histone H2A (H2AK27ub) resulting in a compacted chromatin state (Margueron and Reinberg 2011; He et al. 2012; Gleason and Kramer 2013). The PRC2 complex consists of numerous proteins: two WD40-domain proteins, MULTICOPY SUPPRESSOR OF IRA1 (MSI1) and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE); one of SET-domain proteins: CURLY LEAF (CLF), SWINGER (SWN) or MEDEA (MEA); and one of VEFS-domain proteins: VERNALIZATION2 (VRN2), FERTILIZATION-INDEPENDENT SEED2 (FIS2) or EMBRYONIC FLOWER2 (EMF2). Current data available

for *Arabidopsis thaliana* suggest the existence of different PRC2 complexes in plants: VRN-PRC2, FIS-PRC2 and EMF-PRC2. VRN-PRC2 promotes flowering after vernalization by silencing the expression of the flowering repressor *FLOWERING LOCUS C* (*FLC*) (Gendall et al. 2001; Jiang et al. 2008; Butenko and Ohad 2011). FIS-PRC2 is the main complex involved in regulation of gametophyte and endosperm development. It is responsible for inhibiting the endosperm development in the absence of fertilization. FIS-PRC2 also suppresses the endosperm and embryo cell proliferation after fertilization (Guitton et al. 2004; Köhler and Makarevich 2006). EMF-PRC2 regulates the transition from the vegetative to the generative phase through suppression of important flowering regulators (Yoshida et al. 2001; Chanvivattana et al. 2004). Both EMF-PRC2 and VRN-PRC2 complexes can together control the development of the sporophyte and have a major role in ensuring differentiation and repressing stem cell genes (Bemer and Grossniklaus 2012). The loss of *Arabidopsis MSI1* function results in numerous disorders in the vegetative and embryogenic development, leads to defects in shoot apical meristems, floral meristems, primordia and embryo abortion at different developmental stages (Hennig et al. 2003; Köhler et al. 2003; Bouveret et al. 2006; Schönrock et al. 2006; Steinbach and Hennig 2014). In *msi* mutant plants displayed defects in cell division on early stages of embryogenesis and over-proliferation with deficiency in differentiation of embryos (Köhler et al. 2003; Guitton et al. 2004), indicating an important role of *MSI1* in the zygotic embryo development control. Together with *MSI1, FIE* is an important epigenetic regulator of the reproductive program in *Arabidopsis*. Mutation in the *FIE* gene and the absence of its product results in defects during embryonic development and 50% embryo abortion, similarly to what has been observed in the *msi* mutant (Spillane et al. 2000; Yadegari et al. 2000; Katz et al. 2004). Plants that lost the *FIE* expression show a normal body plan of *A. thaliana* seedlings, but form floral buds very fast and develop copious ectopic cells and organs (Kinoshita et al. 2001; Bouyer et al. 2011).

Information on the contribution of PRC2 to the regulation of somatic embryogenesis induction phase in plants is very scarce. It is only *Arabidopsis* mutants with reduced or inhibited expression of PRC2 complex components that have been studied. These mutations revealed unorganized cell divisions and callus-like tissue development with ability to form somatic embryos (Chanvivattana et al. 2004; Makarevich et al. 2006; Bouyer et al. 2011; Ikeuchi et al. 2015; Mozgova et al. 2015).

To date, identification of *LEC1, L1L* and genes coding protein components of the PRC2 complex and their expression during the induction phase of *Medicago truncatula* SE has not been described. Therefore, the work reported here was aimed at investigate the changes in the expression of two somatic embryogenesis marker genes (*LEC1, L1L*) and genes from the PRC2 complex (*CLF, SWN, FIE, MSI1, VRN2*) genes during the SE induction phase comparing their expression in two genotypes of *M. truncatula* cv. Jemalong: the embryogenic (M9-10a) and the non-embryogenic (M9) line, which could allow obtaining new insights into the involvement of these genes into SE regulation.

Materials and methods

Plant material

Seeds of two genotypes of *Medicago truncatula* cv. Jemalong: the high embryogenic (M9-10a) and the non-embryogenic (M9) line, were kindly provided by Pedro Manuel Fevereiro from Instituto de Tecnologia Quimica e Biologica (ITQB), Portugal. The seeds were used to produce mother plants. Before sowing, the seeds were scarified in 2 ml Eppendorf tubes in 1 ml H_2SO_4 (96%) on ice and shaken for 8 min. After scarification, the seeds were rinsed ten times in cold sterile water and immersed for 30 min in 1 ml gibberellin A_3 (100 µM) solution, then rinsed once with sterile water. Next, the seeds were placed on sterile 15 cm Petri dishes (100 seeds per dish) lined with moisturized filter paper. They were stratified in darkness at 4 °C for 2 days and then transferred to 20 °C for 1 day. Those seeds with well-developed embryonic root (1 cm) were seeded into a mixture of sand, soil, perlite and vermiculite (1:1:1:1). The plants were grown in a growth room at 24 ± 1 °C under a 16 h photoperiod of 70 µM m^{-2} s^{-1} GreenLED (Philips).

Tissue culture protocol

Initial explants for callus induction in both lines of *M. truncatula* were obtained from well-developed trifoliate leaves from the second to third nodes of 60 day-old mother plants. The leaves were surface-sterilized in 1% sodium hypochlorite (using Domestos) for 5 min and then rinsed three times in sterile water. For callus formation induction, three square-shaped explants of 1 cm side length were cut off from each trifoliate leaf and placed on Petri dishes (ø 55 mm) filled with the SH medium (Schenk and Hildebrandt 1972) supplemented with 0.5 µM 2,4-D and 1 µM zeatin with 30 g l^{-1} sucrose. The medium was solidified with 2.5 g l^{-1} gerlit and adjusted to pH 5.7. The culture was kept at 28 ± 1 °C in darkness for 21 days. Subsequently, the callus tissue was transferred to the MS differentiation medium (Murashige and Skoog 1962) with 30 g l^{-1} sucrose and 2.5 g l^{-1} gerlit, pH 5.8. The explants were cultured for 14 days at 24 ± 1 °C under a 16 h photoperiod of 70 µM m^{-2} s^{-1} GreenLED (Philips).

RNA isolation and cDNA synthesis

Samples were collected at four time points (day 2, 7, 14, and 21) during the SE induction phase in both lines, except for day 0 when leaves from intact plants were used. Three biological samples, each consisting of 7 trifoliate explants representing each time point pooled together were subjected to RNA isolation. Total RNA was isolated from 50 mg of frozen tissues in 1 ml TRIzol Reagent (ZymoResearch) using Direct-zol™ RNA-MiniPrep Kit (ZymoResearch) according to the manufacturer's instructions. DNA contamination was removed by using DNase I (ZymoResearch). RNA was eluted in 30 µl DNase\RNase Free-water. The purity and concentration of RNA was checked with BioSpec-nano (Shimadzu) and by electrophoresis in 2% agarose gel. First-strand cDNA of each sample was synthesized from 500 ng total RNA in a 20 µl reaction volume using the High-Capacity cDNA Reverse Transcription Kit (LifeTechnologies) according to the appropriate protocol and then used for quantitative PCR (qPCR).

Sequence analysis

To perform *Medicago truncatula* gene identification, *Arabidopsis thaliana* reference amino acid sequences of *LEC1, L1L*, and *PRC2* genes were obtained from the TAIR data base (http://www.arabidopsis.org/) and used to BLAST search against the JCVI Mt 4.0v1 data base (http://www.jcvi.org/medicago/) of *M. truncatula*. The candidate genes are summarized in Table 1. Locations of specific domains were confirmed in the InterPro data base. The sequence alignments analysis was used to check the homology of protein sequences. The similar protein sequences obtained from the NBCI data base for *Arabidopsis thaliana, Brassica napus, Cicer arietinum, Glycine max, Medicago truncatula, Nicotiana tomentosiformis, Theobroma cacao, Phaseolus vulgaris, Ricinus communis, Solanum lycopersicum*, and *Vitis vinifera* were used to perform ClustalW alignments. The sequence alignments analysis was performed using the Geneious 6.1 software (http://www.geneious.com, Kearse et al. 2012). Phylogenetic unrooted trees were building with the Neighbor-Joining method and Jukes-Cantor genetic distance model. The trees were resampled 1000 times using the bootstrap method.

Quantitative real-time PCR

Gene-specific primers for quantitative real-time PCR (qPCR) were designed using the PrimerExpress® Software v3.0 (LifeTechnologies). All the sequences and parameters are given in Table 1. qPCR was performed with the SYBR Select Master Mix (Applied Biosystems) using the STEP ONE Real-time PCR System (LifeTechnologies) following the manufacturer's instructions. The 10 µl reaction mixture contained 5 µl SYBR Select Master Mix, 0.2 µl 10 mM primers, 1 µl cDNA template, and 3.8 µl DNase/RNase-free distilled water. The expression profile of selected genes in the M9 and M9-10a lines during the SE induction phase was performed using 1:5 cDNA dilution. Analyses for both lines were run on separate plates. Additionally, the Inter-Plate Calibrator analysis was performed on each plate according to the GenEX user guide to compare profiles on one plot. To confirm the changes in transcription level at day 7 and 14, 1:3 cDNA dilutions was used and the analyses for both lines were performed on a single plate. Three biological replicates of each time point and three technical ones were analyzed. The qPCR reaction conditions were as follows: initiation at 95 °C for 2 min, followed by 40 cycles of amplification with 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing. The final extension was performed at 60 °C for 1 min. The dissociation curves were analyzed to check for gene-specific amplification; no unspecific products were detected. The reaction efficiency was 95–100%, as tested using a standard curve for each primer pair. Based on the existing bibliography (Kakar et al. 2008; Mantiri et al. 2008; Peréz et al. 2015) selected 5 candidate reference genes and constructed onsite geNorm and NormFinder evaluation within them (supplement 1). For further analysis used *ACTIN2* as reference gene. For each gene, the relative transcript abundance was calculated and expressed as factor change using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), normalized to *ACTIN2* and relative to the lowest observed transcription (for day profiles) or relative to expression in the non-embryogenic line M9 (for distinct day comparisons). Computer analyses were performed using the GenEX software (MultiD Analyses AB, Sweden).

All the experiments were carried out in triplicate. Change in gene expression among induction phase days were analyzed using the GenEX software (MultiD Analyses AB, Sweden). The results are expressed as mean ± SD. Statistical analyses were performed using the Student's t-test and ANOVA. Differences between the mean values were considered to be significant at $p < 0.01$ or $p < 0.05$.

Results

Identification of *LEC1*, *L1L*, and *PRC2* complex genes in *Medicago truncatula*

Prior to the expression analysis of *LEC1, L1L*, and *PRC2* genes it was necessary to carry out the sequence alignment analysis of proteins encoded by these genes.

Analyses of LEC1 and L1L were performed using their amino acid sequences. The phylogenetic tree showed that

Table 1 Description of analyzed genes, their primer sequence and product size

Gene name	Gene short	*Arabidopsis thaliana* accession number	Accession numer nucleotide/ protein	Genome identifier	Primer sequence	Lenght (bp)	Tm (°C)	Amplicon lenght (bp)
Leafy Cotyledon1 - Like	*L1L*	NP_199578.2	XM_013603204	Medtr4g133952	F: AGATGAACACGAGGCAGCAAGT	22	60.97	100
			XP_013458658		R: GCAATTGGCATAAAACGGTCTT	22	59.99	
Leafy Cotyledon1	*LEC1*	NP_173612.2	XM_003589718	Medtr1g039040	F: AGTGAAGGTGAACCTGCTTCTGT	23	59.12	101
			XP_003589766		R: GGCATTGATGAAAACGATGAAGA	23	60.93	
Curly Leaf	*CLF*	NP_179919.1	XM_003611648	Medtr5g016870	F: CGAAGAGTCAATGCCGAAGTC	21	59.63	101
			XP_003611696		R: AGCTGACCCAACAGTTCCTACAA	23	59.84	
Swinger	*SWN*	NP_567221.1	XM_003591348	Medtr1g086980	F: CAAGCATCAAGATTCCACGTATG	23	59.29	101
			XP_003591396		R: ATTGATCGTCAGCCATTCTCTGA	23	60.74	
Vernalization2	*VRN2*	NP_974563.1	XM_003611313	Medtr5g013150	F: GCTTTAAGGGTTTGCGATTTCA	22	60.21	101
			XP_003611361		R: CGTTCACTGCTTGGTAATCATTG	23	59.33	
Multicopy Suppressor of Ira1	*MSI1*	NP_200631.1	XM_003608461	Medtr4g096880	F: TCTCATGCTCGCTCAAGTTCAA	22	60.82	101
			XP_003608509		R: AACCCTCCAACTTCAGGACGAT	22	60.85	
Fertilization Independent Endosperm	*FIE*	NP_188710.1	XM_013610904	Medtr1g028310	F: CGCAGCCGACATACTTCAGAA	21	61.07	101
			XP_013466358		R: GCACATGCCTTGAAATGGAAAT	22	60.82	

LEC1 and L1L to separate into two distinct clades (Fig. 1). *Medicago truncatula* LEC1 formed one clade alongside other LEC1 from the family *Fabaceae: Cicer arietinum, Glycine max* and *Phaseolus vulgaris*. The *Mt*LEC1 showed a 69% identity with *At*LEC1. *Mt*L1L, too, formed one clade alongside other LEC1 from the family *Fabaceae*, but the *Mt*L1L similarity to *At*L1L was 61%.

On the phylogenetic tree SWN and CLF to grouped separately (Fig. 2a). Both SWN and CLF assembled together with the *Fabaceae* proteins examined. *Mt*SWN was more closely related to *At*SWN than to *At*CLF. *Mt*CLF showed a 47% identity with *Mt*SWN. *Mt*MSI1 was a close ortholog of *At*MSI1 (Fig. 2b). Both MSI1 and FIE grouped into one clade with MSI1 and FIE of *Cicer arietinum, Glycine max*, and *Phaseolus vulgaris*, respectively (Fig. 2b, c). The *Mt*FIE amino acid sequence showed a 72% affinity to *At*FIE.

The presence of a protein-specific domain indicates its membership in a particular group of PRC2 complex. The domain-searching analysis revealed that, among the

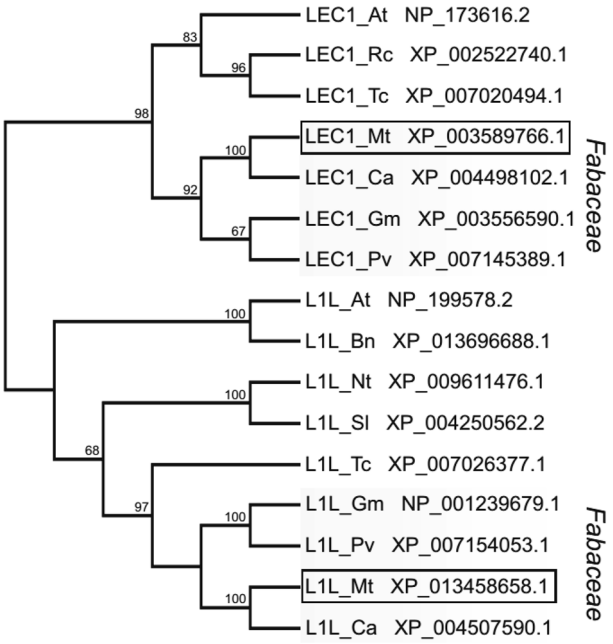

Fig. 1 Unrooted phylogenetic tree based on deduced amino acid sequences of *Medicago truncatula* LEC1 and L1L. *At Arabidopsis thaliana*, *Bn Brassica napus*, *Ca Cicer arietinum*, *Gm Glycine max*, *Mt Medicago truncatula*, *Nt Nicotiana tomentosiformis*, *Tc Theobroma cacao*, *Pv Phaseolus vulgaris*, *Rc Ricinus communis*, *Sl Solanum lycopersicum*

Medicago PRC2 proteins analyzed, only CLF and SWN (described also as EZA1) have the SET domain (Fig. 3). Both MSI1 and FIE contain the WD40 domain, but differ in the number of WD-40 repeats and the presence of the RBBP4 domain in the MSI1 protein.

Callus induction in Jemalong M9 and M9-10a lines and its ability to form the embryos

The callus development in leaf explants of two *Medicago truncatula* cv. Jemalong genotypes, the non-embryogenic (M9) and embryogenic (M9-10a), after 2, 7, 14 and 21 days on the SH induction medium and the somatic embryos production after 14 days on the MS differentiation medium are shown in Fig. 4. During the first two days, the explants became swollen, but no visible differences between the two lines were observed. After a one-week induction the explants curled up at the cut edges and produced callus tissue (Fig. 4). The weight of the callus obtained from the M9-10a explants was 1.5-fold higher than that of the M9 line (Fig. 5). The last two weeks were the period of a rapid callus growth. The M9 leaf explant calluses had compact and even structure with green-yellow colour, while the M9-10a explant callus texture was granular, very loose, and had a light-yellow colour (Fig. 4). After 14 days, the M9-10a line leaf explants produced nearly twice as much callus as the M9 line did (Fig. 5). On day 21, all the M9-10a leaf explants were covered by the callus tissue, whereas the M9 leaf explants showed callus to have developed less expansively (Fig. 4). The weight of the callus obtained from the M9-10a explants was more than two-fold higher than that of the M9 line (Fig. 5). The 21 day-old explants with well-developed callus tissue were placed on hormone-free MS differentiation medium. After the subsequent two weeks, somatic embryos developed only on the M9-10a embryogenic explants.

LEC1 and *L1L* expression during somatic embryogenesis

The relative expression of *LEC1* and *L1L* genes was analyzed during the induction phase at distinct time-points in the non-embryogenic (M9) and embryogenic (M9-10a) lines. In primary explants, the expression level of *LEC1* (Fig. 6a.1) and *L1L* (Fig. 6b.1) was elevated in both lines, and their expression did not change during the entire period in M9. In contrast, expression of *LEC1* in the M9-10a tissues increased: during the last two weeks of induction the expression was 7- and 25-fold higher on days 14 and 21, respectively, compared to the lowest expression observed at day 2. A direct comparison of *LEC1* expression in M9 and M9-10a on day 7 revealed no significant difference between the two lines (Fig. 6a.2). However, the gene's expression in M9-10a was nine-fold higher than that observed in M9 on day 14. The expression profile revealed that transcripts of *L1L* gene was expressed between days 2 and 7 in M9-10a (Fig. 6b.1). The expression grew to reach even 129- and 307-fold increase on days 14 and 21, respectively, compared to the lowest expression in the primary leaf explants of M9-10a. A direct comparison of *L1L* expression in M9 and M9-10a on day 7 and 14 showed a 129- and 168-fold increase in M9-10a, respectively, compared to the expression in M9 normalized to 1 (Fig. 6b.2). Both genes are expressed in embryogenic calluses during the induction phase only; moreover, both are highly expressed during the last two weeks of the process.

Expression analysis of genes encoding PRC2 complex proteins

Since PRC2 is known to take part in transition from the vegetative to the generative phase in *Arabidopsis thaliana* ontogenesis, it was interesting to find out whether the proteins: CLF, SWN, VRN2, MSI1 and FIE, belonging to this complex, are putative involved in acquisition of embryogenic competence during the SE induction phase in *M. truncatula*. The relative expression of genes encoding these proteins was analyzed during the induction phase at distinct time-points. The expression in the M9-10a

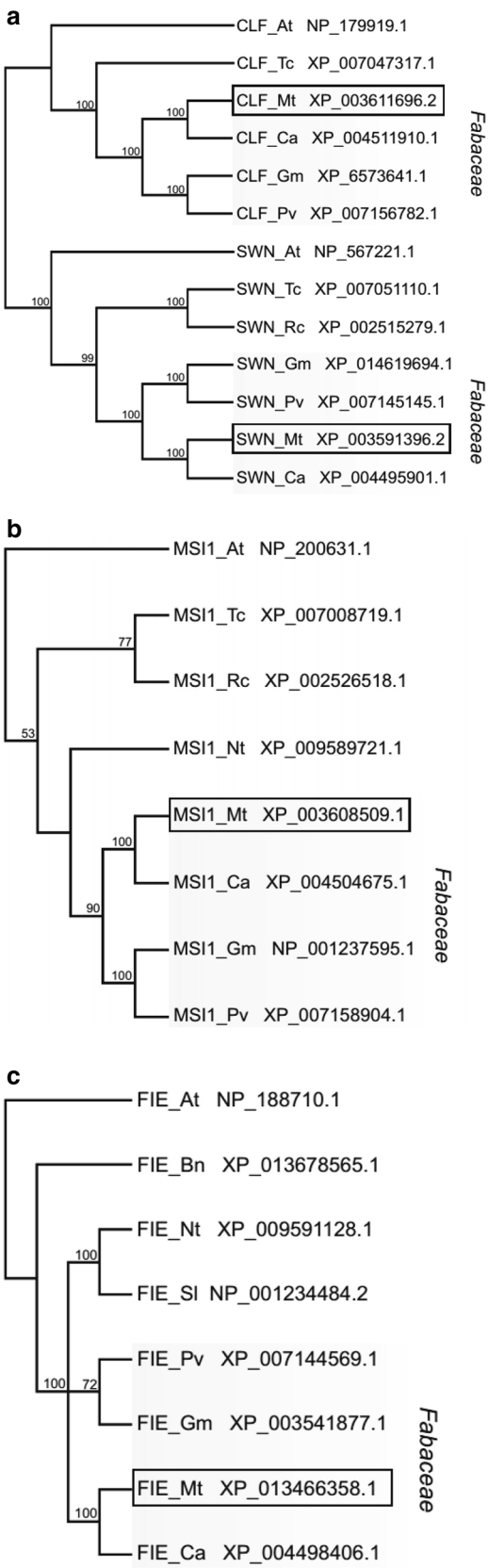

Fig. 2 Unrooted phylogenetic tree based on deduced amino acid sequences of *Medicago truncatula* CLF and SWN (**a**), MSI1 (**b**) and FIE (**c**). *At Arabidopsis thaliana*, *Bn Brassica napus*, *Ca Cicer arietinum*, *Gm Glycine max*, *Mt Medicago truncatula*, *Nt Nicotiana tomentosiformis*, *Tc Theobroma cacao*, *Pv Phaseolus vulgaris*, *Rc Ricinus communis*, *Sl Solanum lycopersicum*

primary explants (day 0) in all the relative gene profiling experiments was assumed to be 1. The *CLF* expression in M9 during induction was slightly up-regulated with regard to that on 0 day (Fig. 7a.1), while the *CLF* expression in M9-10a was highly up-regulated; it was three-fold higher (at day 7 and 14) than the expression in the primary explant. After one- and two-week long induction (days 7 and 14), the expression was 1.8- and 1.6-fold higher in M9-10a (Fig. 7a.2). The expression profiles for *SWN* in both lines (Fig. 7b.1) revealed an similar pattern with a distinct drop down on the second day of induction. Moreover, the expression level of *SWN* on days 7 and 14 remained the same in the two lines (Fig. 7b.2). The expression profiles of *VRN2* and *FIE* genes were very similar in the two lines analyzed (Fig. 7c.1, d.1). The expression during first week of induction increased four- and six-fold for *VRN2* and *FIE*, respectively. From 7 day their expression was on this same level in both lines (Fig. 7c.2, d.2). The expression profile of *MSI1* (Fig. 7e.1) in M9 remained on a similar level, whereas in M9-10a it was highly up-regulated, the expression being 11-, 9-, and 14-fold higher (for days 7, 14, and 21, respectively) than it was at the beginning of induction. Comparison of the *MSI1* expression in both lines on days 7 and 14 showed that in M9-10a was twice as high as that in M9 (Fig. 7e.2). It is worth pointing out that the expression of *VRN2, MSI1* and *FIE* denoted in the primary leaf explants was about three-fold higher in M9 than in M9-10a, which might be regarded as a crucial initial background sufficient to regulate the subsequent processes.

Discussion

The ability to produce embryos from somatic cells of plant explants, known as the somatic embryogenesis (SE), has been studied for a long time, but still remains incompletely understood. At present, only just residual data concerning the regulation of the process at the epigenetic level carried out by the Polycomb proteins are available, and all concern *Arabidopsis*. It is known that acquisition of embryogenic competency is related to transition from the somatic to the embryogenic state with activation of transcription factors among other from the LEC group which the prominent function is the specification of cotyledon identity (Altamura et al. 2016). Since the Polycomb Repressive Complex 2 (PRC2) proteins takes part in transition from the vegetative

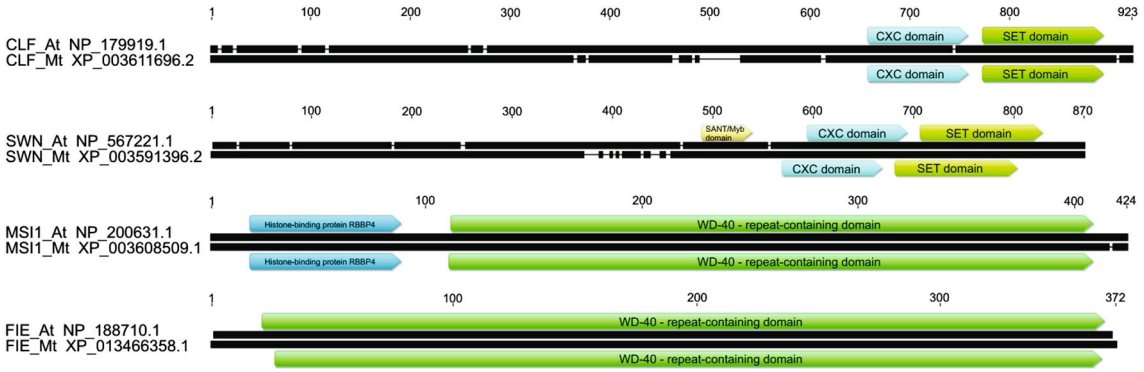

Fig. 3 A schematic view of the domain location of Polycomb Repressive Complex 2 proteins of *Arabidopsis thaliana* (At) and *Medicago truncatula* (Mt)

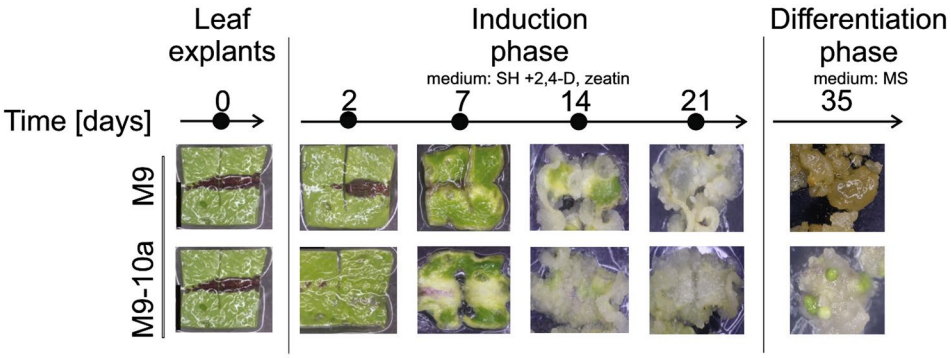

Fig. 4 The leaf-to-callus transition on SH induction medium (21 days) and somatic embryo production on MS differentiation medium (14 days) during the somatic embryogenesis of *Medicago truncatula* non-embryogenic (M9) and embryogenic lines (M9-10a) (*black dots* on the timeline—sampling time points)

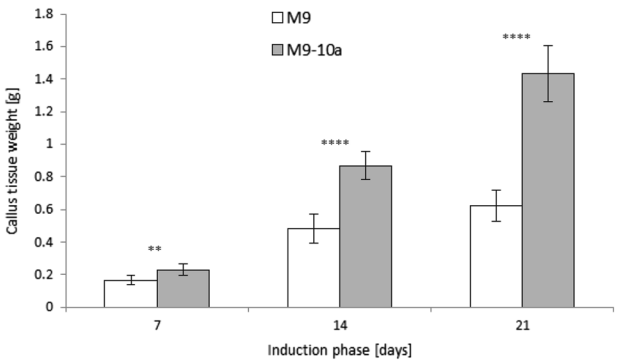

Fig. 5 The growth rate of callus on leaf explants of *Medicago truncatula* non-embryogenic (M9) and embryogenic (M9-10a) *lines* during the induction phase on SH medium

to the reproductive program in *Arabidopsis* (Mozgova et al. 2015), it was interesting to investigate both the expression of genes encoding these proteins and coding the transcription factors from the LEC group during the SE induction in *Medicago truncatula* cv. Jemalong.

Two lines: the non-embryogenic (M9) and embryogenic (M9-10a) were examined during a three-week long induction phase. The first week of induction ended with the residual callus on cut edges of the embryogenic line explants; the changes visible during that period were slight only. Dedifferentiation and the formation of totipotent cells during the initial days is an effect of molecular mechanisms and activation of required transcription factors without any remarkable morphological changes (Rose et al. 2013). During the last two weeks of induction, a rapid growth of callus was observed primarily in the embryogenic line, but the difference in the callus structure between the two lines was not an obvious factor determining the embryogenic potential. It would be more appropriate to call this stage of the process the expression phase (according to Almeida et al. 2012) rather than the induction one. When calluses were transferred to a hormone-free medium, the somatic embryos started to develop on the embryogenic line explants only, which resulted in phenotypes strong enough to differentiate between the lines tested. To sum up, the induction phase in the embryogenic line involves two distinct stages: dedifferentiation and expression, but it is not possible to determine when the formation of totipotent cells starts or ends.

Several genes are known to be involved in SE induction in plants, the most interesting among them being the *LEC*-group genes. The sequence alignment analysis of LEC1 and L1L in *Medicago truncatula* confirmed that, like in other

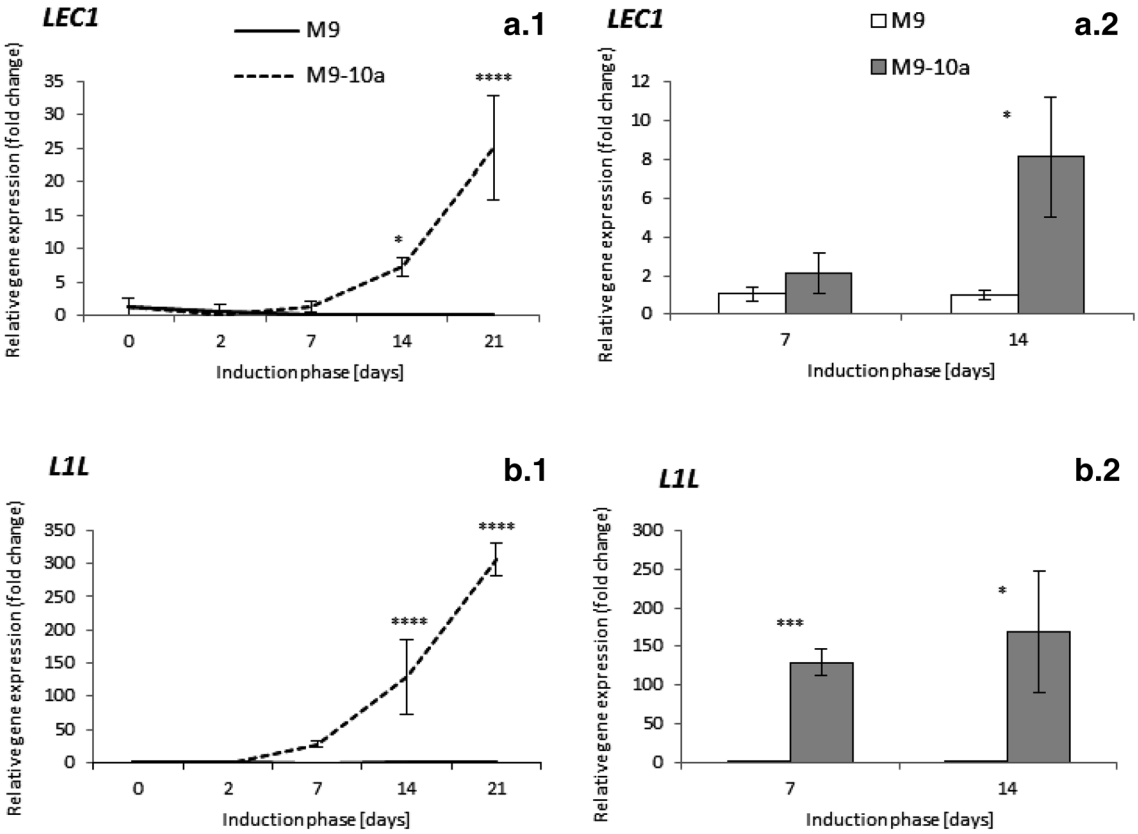

Fig. 6 Relative expression patterns of *LEC1* and *LEC1-LIKE* genes in the leaf explants of *Medicago truncatula* non-embryogenic (M9) and embryogenic (M9-10a) lines during the induction phase on SH medium. Transcript levels for *LEC1* and *LEC1-LIKE* genes were estimated by Real-time PCR and normalized to that of *ACTIN2*. *Bars* show standard deviation. *Dotted line* indicate and *white bars* M9-10a *line, solid line* and *grey bars*-M9 line. **a–b.1** Line graphs show the dynamic expression of particular genes during the 21 day of induction phase and indicates the fold changes of its expression relative to the lowest value for which assumed value of 1. Statistical analyses, two-way ANOVA with confidence interval 0.05, significance between groups indicated as * for $P \leq 0.05$, ** for $P \leq 0.01$,*** for $P \leq 0.001$ and **** for $P \leq 0.0001$. *Bars* indicate ±SD. **a–b.2** Column graphs show the difference in the level of transcription at 7 and 14 day between M9 and M9-10a line expressed as fold changes in relation to the obtained value in M9 line taken as 1. Statistical analyses, two-tailed t-test with confidence interval 0.05. *Asterisks* represent significance level respectively: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$. *Bars* indicate ± SD

vascular plants, these transcription factors grouped into two separated clades on phylogenetic tree, despite their overlapping role (Cagliari et al. 2014). The expression of *L1L* was observed to be induced only in the embryogenic line during the first week of induction and continued to increase steeply for the next two weeks, while *LEC1* was only just expressed during this latter period. Moreover, the expression level of *L1L* was much higher than that of *LEC1*. As both transcription factors can be functionally substituted to some extent, it is not clear if the presence of both is necessary during SE. The expression profile indicates that activation of both genes overlapped with the expression phase, which might suggest that the genetic program differs from that of the dedifferentiation phase. According to Huang et al. (2015), the high expression of *LEC1* during *Arabidopsis* zygotic embryogenesis is caused by the release of the vegetative tissue from the suppressing environment, which might suggest that during the first two weeks of *Medicago truncatula* SE induction a switch from the vegetative to embryonic program takes place. Furthermore, our results indicate that *LEC1* and *L1L* may be used as good gene markers for SE in *Medicago truncatula*.

The Polycomb Repressive Complex 2 belongs to epigenetic factors which, together with PRC1, play a key role in plant development by acting through the regulation of gene silencing. Two members of the PRC2 complex: CLF and SWN, belong to the SET-domain proteins involved in histone H3 lysine 27 trimethylation. They are active in meristems and dividing cells throughout the plant vegetative and reproductive development (Chanvivattana et al. 2004). The phylogenetic tree of *Arabidopsis thaliana* and *Vitis vinifera* showed that CLF and SWN belonged to two distinct clades, but were much more similar to each other that to another SET-domain protein, MEDEA (MEA) (Chanvivattana et al.

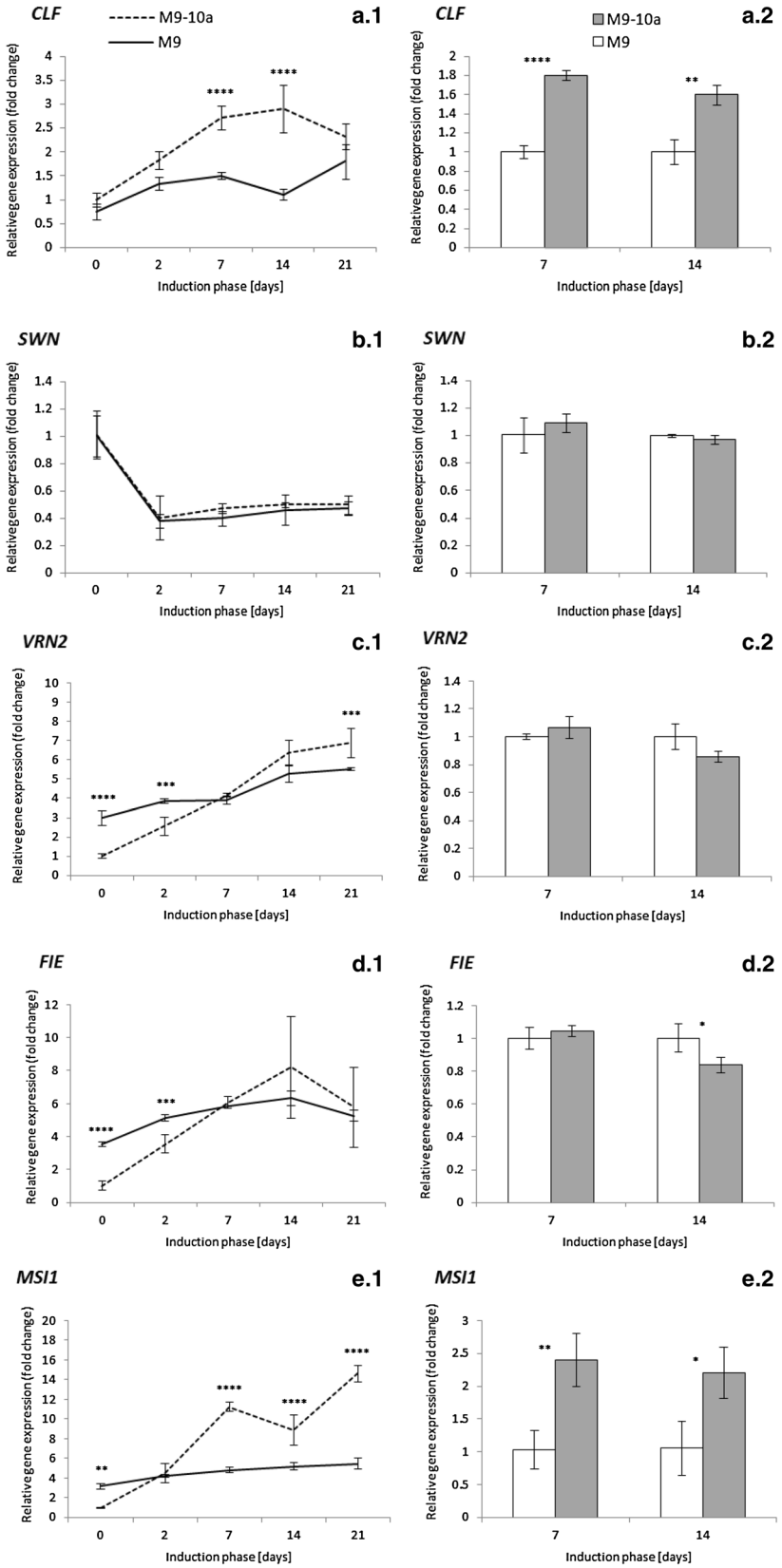

Fig. 7 Relative expression patterns genes of the *PRC2* complex in the leaf explants of *Medicago truncatula* non-embryogenic (M9) and embryogenic (M9-10a) lines during the induction phase on SH medium (**a**–**e**). Transcript levels for each gene were estimated by Real-time PCR and normalized to *ACTIN2*. *Bars* show standard deviation. *Dotted line* indicate and *white bars* M9-10a *line, solid line* and *grey bars*—M9 line. **a–b.1** Line graphs show the dynamic expression of particular genes during the 21 day of induction phase and indicates the fold changes of its expression relative to the lowest value for which assumed value of 1. Statistical analyses, two-way ANOVA with confidence interval 0.05, significance between groups indicated as * for $P \leq 0.05$, ** for $P \leq 0.01$,*** for $P \leq 0.001$ and **** for $P \leq 0.0001$. *Bars* indicate ± SD. **a–b.2** Column graphs show the difference in the level of transcription at 7 and 14 day between M9 and M9-10a line expressed as fold changes in relation to the obtained value in M9 line taken as 1. Statistical analyses, two-tailed t-test with confidence interval 0.05. *Asterisks* represent significance level respectively: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$. *Bars* indicate ±SD

2004; Almada et al. 2011). The analysis of *M. truncatula* CLF and SWN showed, too, that these two proteins grouped separately. *Mt*MEA was excluded from the sequence alignment analysis due to the lack of *At*MEA close orthologous in any available *Medicago truncatula* data base. MEA was shown to occur in the family *Brassicaceae* only (Spillane et al. 2007). Recent research revealed that CLF and SWN might be essential for the callus tissue formation in plants. During the SE induction in *M. truncatula, CLF* expression increase significantly in consecutive days of the process in the embryogenic line (M9-10a). In the non-embryogenic line (M9), expression of this gene was stable in all the stages samples analyzed. A completely different pattern of gene expression was shown by *SWN*: its transcription dropped sharply between days 0 and 2, and remained at low levels until the end of the induction. This result suggests that the increase of the *CLF* expression overlapped with the dedifferentiation of somatic cells, callus growth and acquisition of the embryogenic potential. In our case, the expression of *SWN* decreased, while that of *CLF* increased during induction in the embryogenic line. These results are consistent with previous conclusions that CLF and SWN may play interchangeable roles in the PRC2 complex of *Arabidopsis* (Chanvivattana et al. 2004). Also Chanvivattana et al. (2004) found that the lack of expression of the *SWN* and *CLF* genes led to spontaneous emergence of a callus-like structure on whole seedlings. However, a different result was reported by He et al. (2012), where *Arabidopsis thaliana* double mutants *clf/swn* failed to develop the callus tissue from leaf explants, but it was observed on root explants. Those suggest that these genes are required for the leaf, but not the root and seedling, during callus formation. Probably, CLF is also an active component of PRC2 during the SE induction phase in *Medicago truncatula*.

The VRN2 protein is another component of the PRC2 complex and, together with other proteins, may form a VRN-PRC2 complex. It regulates the transition from the vegetative to the reproductive development (Bemer and Grossniklaus 2012). During the induction phase, the *VRN2* gene expression increased gradually with the strong callus tissue development in the embryogenic line (M9-10a) of *M. truncatula*, whereas the expression in the non-embryogenic line (M9) was stable and the weight of the callus was lower than that in the M9-10a line. Different results were obtained during the development of *Arabidopsis emf2/vrn2* mutants which the expression of these genes during the callus-like tissue formation was absent (Schubert et al. 2005).

The two other components of the PRC2 complex, i.e. MSI1 and FIE bind together with a high efficiency and can work as a complex (Köhler et al. 2003). The amino acid identity and the number of WD repeats observed in MSI1 and FIE confirmed close identity of the protein in *M. truncatula* and *A. thaliana*. The two lines of *M. truncatula* tested: the embryogenic and the non-embryogenic differed in their *MSI1* expression during the induction phase of somatic embryogenesis. An increased expression of the gene was observed in the embryogenic line's callus tissue, which results in embryo formation during the differentiation phase. On the other hand, the *MSI1* transcript level was stable in the non-embryogenic line and embryos did not appear. A strong increase in this gene's expression during the first week of the induction phase in the embryogenic line may suggest its role in a switch from the vegetative to the embryonic development pattern. These results may suggest that *MSI1* is required for explant somatic cells to acquire the embryogenic potential and for embryo development, as in zygotic embryogenesis. In our experiment expression of FIE was on this same level in M9 and M9-10a line. Also studies on *fie* mutants showed that, they have the ability to disorganized growth, to form callus-like structures, and to develop somatic embryos at a high frequency (Kinoshita et al. 2001; Bouyer et al. 2011).

Two components of PRC2 complex, CLF and SWN are known to insert H3K27me3 marks in histones. Analysis of mutants showed that the lack of *clf/swn* activity resulted in an up-regulation of *LEC1* and *LEC2* genes in *Arabidopsis thaliana* (Makarevich et al. 2006). Regulation of *LEC1* by epigenetic mechanisms was also proposed for *Coffea camephora* (Nic-Can et al. 2013) where the presence of H3K27me3 marks within the *LEC1* histone-associated region was a possible cause of reduced transcriptional activity of the gene during early stages of induction somatic embryogenesis. In these experiments, *LEC1* expression increased with progress of somatic embryogenesis induction, which was consistent with our results where *LEC1* and *L1L* transcripts started to appear between days 7 and 21. This might be the effect of histone demethylation carried out by Trithorax group proteins, which removed H3K27me3 marks inserted by PRC2 proteins (Pien and Grossniklaus 2007; Köhler and Hennig 2010). This hypothesis, however, needs to be experimentally tested. Another possible mechanism might be related to differences of *PRC2* genes expression in the primary explants. *VRN2, MSI1* and *FIE* showed a lower expression in the embryogenic line leaf explants, which might have resulted in the ability to form embryogenic callus. The higher expression in the non-embryogenic line may play an inhibitory role in this process, so - because of the difference in the expression of these genes—primary explants may possess a different level of chromatin trimethylation and a changed ability to step in SE. Involvement of *FIE* and *MSI1* genes in determining the H3K27me3 level has been confirmed in *A. thaliana* (Bouyer et al. 2011; Derkacheva et al. 2013).

Our results showed that in leaf explants of *Medicago truncatula* embryogenic line during the prime events of somatic embryogenesis (induction phase) a strong

up-regulation of *LEC1* and *L1L* genes with accompanied increased of *CLF* and *MSI1* expression.

Acknowledgements This work was partly supported by the National Scientific Centre (NCN) Grant No. NN303801340. We are indebted to dr Teresa Radziejewska for linguistic assistance.

Author contributions AO, RI and KŁ conducted experiments and carried out the statistical analysis. EK contributed to the discussion of result. AO and EK wrote the manuscript, RI participated in the writing part of the manuscript. All the authors read the final version of the paper.

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